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Effect of Formulation and Application Method on the Efficacy of Aerial and Submerged Conidia of *Metarhizium flavoviride* for Locust and Grasshopper Control

Nina E. Jenkins[a]* & Matthew B. Thomas[b]

[a] International Institute of Tropical Agriculture (IITA), Biological Control Centre for Africa, BP08-0932 Cotonou, Republic of Benin
[b] Leverhulme Unit for Population Biology and Biological Control, Natural Environmental Research Council Centre for Population Biology, Imperial College, Silwood Park, Ascot, Berks SL5 7PY, UK

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Abstract: A study was carried out to investigate the relative infectivity of aerial and submerged conidia of *Metarhizium flavoviride* to *Schistocerca gregaria* and *Zonocerus variegatus*. The effect of formulation and application method on initial infectivity and field persistence of these conidia was investigated.

Strain IMI 330189 was highly virulent to *S. gregaria* but showed relatively low virulence to *Z. variegatus*. Direct contact with conidia from the initial spray application resulted in 100% mortality of *S. gregaria* for all formulation and application combinations. The mean survival time of infected locusts was significantly shorter for treatments using a knapsack sprayer containing submerged conidia in water plus 10 ml litre^{-1} 'Codacide'® (seven days), than treatments with aerial conidia in oil using ULV techniques (8.9 days) or submerged conidia in modified (water plus adjuvants) ULV (MULV) (nine days) or in water-based (VLV) applications (9·3 days).

Both aerial and submerged conidia persisted long enough in the environment to effect significant mortality via secondary pick-up of spray residue from vegetation. Persistence was greatest in the ULV and MULV treatments, where the oil component of the formulations provided greater protection of the conidia from environmental stresses. The consequences of secondary pick-up of conidia from the different treatments on total mortality from a single application were examined using a simple host–pathogen model. This predicted that the ULV treatment would be much more effective than the other treatments under conditions where direct contact with the spray was limited.

The results of these investigations are discussed in the context of development of optimum spray strategies for control of locusts and grasshoppers, and other pests, under different environmental conditions.

Key words: *Schistocerca gregaria*, *Zonocerus variegatus*, *Metarhizium flavoviride*, formulation, persistence, infectivity.

* To whom correspondence should be addressed at: International Institute of Biological Control, Silwood Park, Buckhurst Road, Ascot, Berks SL5 7TA, UK

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1 INTRODUCTION

The collaborative IIBC/IITA/DFPV (International Institute of Biological Control, of CAB INTERNATIONAL/International Institute of Tropical Agriculture Plant Health Management Division/ Département de Formation en Protection des Végétaux of CILSS, the Comité Inter-État de Lutte contre la Secheresse au Sahel.) research programme for the control of locusts and grasshoppers (LUBILOSA; LUtte BIologique contre les LOcustes et SAuteriaux) has developed a biological insecticide based on an oil formulation of aerial conidia of the entomopathogenic Deuteromycete *Metarhizium flavoviride* Gams & Rozsypal.[1] Field trials using conventional ultra-low volume (ULV) spraying equipment have been conducted successfully on *Zonocerus variegatus* L.,[2,3] *Schistocerca gregaria* Forskål, (O.-K. Douro-Kpindou, pers. comm.; C. Kooyman and I. Godonou, pers. comm.), and a variety of Sahelian grasshoppers.[4]

Ecological studies following application of conidia in a standard kerosene + groundnut oil (70 + 30 by volume) formulation have shown that secondary pick-up of conidia from the spray residue significantly increases total mortality in the field.[4,5] This secondary pick-up is particularly important in areas of dense vegetation, where infection due to direct hit by spray droplets can be $<40\%$,[4,5] and for contacting mobile insects that may disperse into treated areas some days after spraying. Determining the extent of secondary pick-up is therefore important if the overall effects of a spray application are to be quantified. To this end simple insect–pathogen models have proved to be useful tools for interpreting patterns of disease development in grasshopper populations after spraying.[5]

To date, all field applications in this programme have been carried out using aerial conidia formulated in oil and applied at ULV rates. However, the recent discovery that *M. flavoviride* can be induced to produce conidia in submerged culture,[6] presents the opportunity for formulation and application of submerged conidia. Submerged conidia can be produced using conventional deep-tank fermentation, which is considered to be more economical than solid-state fermentation for large-scale production of mycopesticides.[6,7] Submerged conidia are produced from phialides in the same manner as aerial conidia, but differ in their surface properties in that they are hydrophilic and hence do not formulate easily in oils. Preliminary studies on submerged and aerial conidia have shown the two spore types to be of equal virulence to *S. gregaria* when formulated in water (N. Jenkins, unpublished).

Although ULV application techniques have traditionally been used in combination with oil formulations, techniques and equipment have recently been developed for water-based ULV (very low volume (VLV)) and modified (water plus adjuvants) ULV (MULV) applications. The latter two techniques offer the advantage of a low-volume application method for water-based formulations, which would otherwise require the use of conventional high-volume techniques.

In this paper we present the effects of formulation and application method on initial infectivity and field persistence of submerged conidia and compare these with standard ULV applications of aerial conidia formulated in oil.

2 MATERIALS AND METHODS

2.1 Production of conidia

M. flavoviride IMI 330189 ss (single spore isolation) was maintained on potato carrot agar (PCA) slopes at 10°C and was re-isolated from infected adult desert locusts at approximately three-month intervals to maintain virulence. Working cultures of the fungal strain were grown on Sabouraud dextrose agar (SDA) to provide a spore inoculum.

2.1.1 Submerged conidia

Submerged conidia were produced using the method of Jenkins and Prior.[6] Erlenmeyer flasks (250 ml capacity) containing 75 ml of liquid medium, were inoculated with a conidial suspension (1 ml) containing approximately 6×10^6 conidia ml^{-1}. Cultures were incubated on a rotary shaker at 150 rev min^{-1} for six days at approximately 30°C. During harvesting, submerged conidia were separated from mycelium by passing the broth through a 75-μm mesh sieve. Conidia were then left to settle overnight in a refrigerator, before removal of the supernatant and subsequent formulation.

2.1.2 Aerial conidia

Aerial conidia were produced using a standard two-phase production system on rice. Fungal biomass was produced in shake flasks in a liquid medium containing brewers' yeast (20 g $litre^{-1}$) and sucrose (30 g $litre^{-1}$). The resulting biomass was then transferred to par-boiled, sterilised rice contained in autoclavable bags within stackable plastic bowls (400 mm diameter $\times$ 200 mm depth) and left to conidiate over a period of 14 days. The rice and conidia were then air-dried for four days, following which the conidia were sieved from the rice and further dried in a desiccator to a moisture content of 5%. These conidia were stored as a dry powder at 15°C until formulation and use.

2.2 Formulation and application

2.2.1 Preparation of conidia formulations

Experimental investigations were conducted in an area of natural mixed-grass vegetation at the IITA Biological Control Centre, Cotonou, Benin. Four conidial formu-

TABLE 1
Formulation and Application Methods for the Four Spray Treatments. All Formulations were Adjusted to provide the Equivalent of $3{\cdot}5 \times 10^{12}$ Conidia ha^{-1} upon Application

Conidial type	*Formulation*	*Application method and volume application rate*
Aerial conidia	Kerosene : groundnut oil (7 : 3 by volume)	'Micron' Ulva-plus hand-held sprayer (5 batteries, disc speed *c.*5000 rev min^{-1}) equivalent to 2 litre ha^{-1} (ULV)
Submerged conidia	Water plus 100 ml $litre^{-1}$ 'Codacide'®	'Micron' Ulva-plus hand-held sprayer (5 batteries, disc speed *c.*6000 rev min^{-1}) equivalent to 5 litre ha^{-1} (MULV)
Submerged conidia	Water	'Micron' Ulva-plus hand-held sprayer (4 batteries, disc speed *c.*5000 rev min^{-1}) equivalent to 10 litre ha^{-1} (VLV)
Submerged conidia	Water plus 10 ml $litre^{-1}$ 'Codacide'®	Knapsack sprayer equivalent to 200 litre ha^{-1}

lations were prepared and applied according to Table 1. Kerosene/groundnut oil is the standard formulation used within the LUBILOSA project for formulation of aerial conidia. 'Codacide'® (Microcide Ltd, Bury St Edmunds, Suffolk, UK) is a commercial carrier oil, containing 50 ml $litre^{-1}$ emulsifier in rape seed oil; it is promoted as an adjuvant/carrier for use with a wide range of chemical pesticides to aid spreading and sticking of water formulations to the target.

Viability of conidia in each formulation was checked prior to spraying using a standard 24-h germination test on SDA as described by Morley-Davis *et al.*[8] Each treatment was applied to four 10 × 10 m plots arranged in a randomised block design. Plots were separated by 10 m within the blocks and each block was 50–100 m from its nearest neighbour.

2.2.2 Direct spray contact

To assess direct contact rate of the conidia in the different treatments, 20 *S. gregaria* adults and 20 *Z. variegatus* nymphs (predominantly 5th instar) were introduced into temporary field cages ($0{\cdot}5\ m^2 \times 0{\cdot}7$ m high), made from large-mesh nylon netting and placed around the natural vegetation at the centre of each of the plots prior to application. Shortly after application (< 30 min), these insects were removed and maintained in the laboratory to assess disease and mortality levels. To assess the differences in droplet size and density between treatments and to examine the effects of the cages on spray impaction, spray droplets were recorded using water- and oil-sensitive paper (Ciba-Geigy, Basel) for the water and oil formulations, respectively. Four pieces of indicator paper were placed on posts inside each cage (two posts, each with one piece of paper 50 cm and 20 cm above soil surface) and four were placed on posts adjacent to each cage (one post each side of the cage with paper positioned as above).

2.2.3 Infectivity of spray residue

Infectivity of the spray residues was monitored using a field bioassay technique similar to that described in Carruthers *et al.*[9] One field cage ($0{\cdot}5\ m^2 \times 0{\cdot}4$ m high with metal mosquito mesh sides, a removable top and an open bottom) was placed at the centre of each of the sprayed plots one day after treatment and 15 uninfected *S. gregaria* and *Z. variegatus* were placed inside. After 72 h these insects were removed and were incubated in the laboratory for 21 days to monitor mortality and infection. This procedure was repeated 4, 7, 10, and 13 days after spraying with the cages placed in a new position in the sprayed plots on each date.

3 RESULTS

3.1 Direct spray contact

The spray deposits on the indicator cards were assessed using a standardised scoring system developed by Bateman.[10] This technique uses a droplet deposit gauge to score spray deposits and to give a measure of droplet size and density. As expected from the different application volumes and techniques, there was a highly significant difference in droplet size and density between treatments (Kruskall-Wallis test, both $P \ll 0{\cdot}001$). Within treatments, comparison of droplet sizes revealed no significant differences between cards inside and outside the cages (Mann-Whitney U test). However, similar analysis of droplet density data revealed that the cages caused a significant reduction in the number of spray droplets hitting the indicator cards for the MULV and knapsack treatments (both $P < 0{\cdot}01$).

Figures 1(a) & (b), show the cumulative proportional mortality curves for *S. gregaria* and *Z. variegatus* respectively. It can be seen that mortality of *S. gregaria* populations reached 100% in all treatments within 22

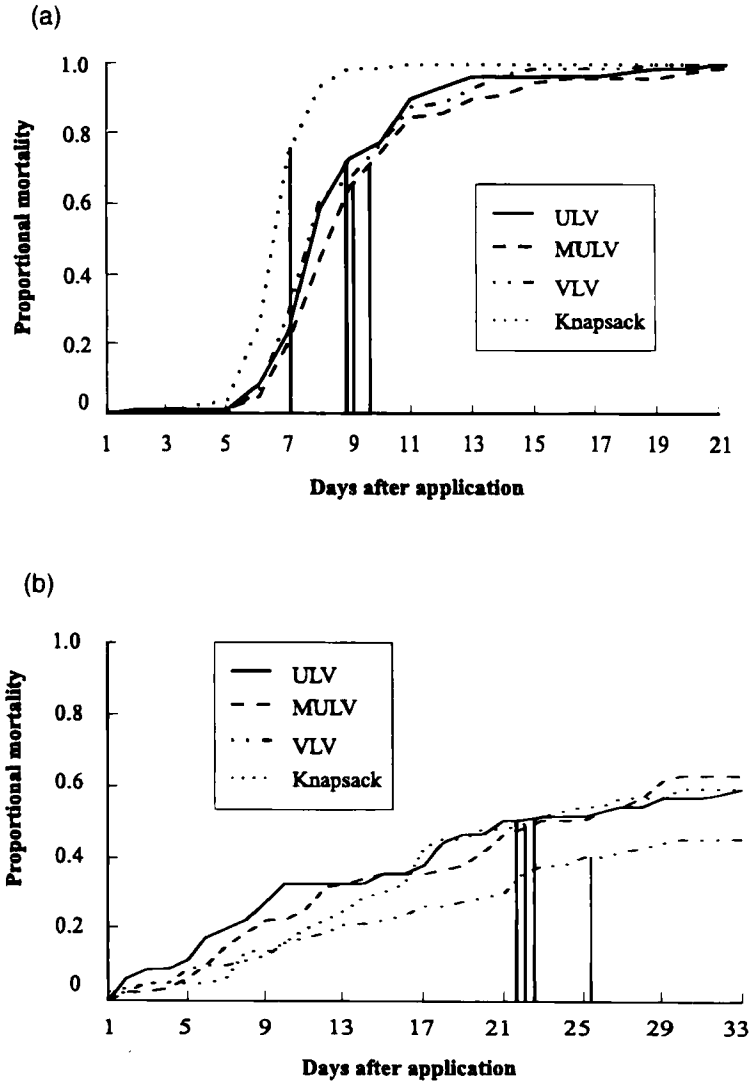

Fig. 1. Mean cumulative proportional mortalities of populations of (a) *Schistocerca gregaria* and (b) *Zonocerus variegatus* following application of conidia of *Metarhizium flavoviride* using ULV, MULV, VLV and knapsack spraying techniques. The vertical lines show mean survival times for each of the spray treatments.

days after spraying. Mortality of *Z. variegatus*, however, approached only 45–63%, after 33 days, with no significant differences between mortality levels in the four application methods. Incubation of the insect cadavers revealed that only *c.*50% of *Z. variegatus* mortality was due to mycosis. In contrast, mycosis of *S. gregaria* cadavers was >97%.

Kaplan-Meier survival analysis (in SPSS for windows) was used to obtain the mean survival time (AST) for each replicate population, thus providing a measure of the rate of mortality for each application method. Randomised block analysis of variance ($\sqrt{}$arcsin transformation of the ASTs expressed as a proportion of the experimental duration) revealed no significant difference between any of the application methods for *Z. variegatus*. For *S. gregaria*, however, the mean AST for the knapsack application (seven days) was significantly lower than for the other treatments (ULV = 8·9, MULV = 9, VLV = 9·3)($F_{3,9} = 5{\cdot}808$; $P = 0{\cdot}0172$) (Fig. 1(a)).

3.2 Spray residue

Unlike direct spray contact, which caused reasonable levels of infection in the *Z. variegatus* samples, the reduced dose of conidia provided by secondary pick-up from the vegetation resulted in negligible infection rates. Therefore, examination of residual infectivity is restricted to the data collected for *S. gregaria*.

The mean proportional mortality and infection of *S. gregaria* populations exposed to the different spray treatment residues are presented in Fig. 2. This figure shows the levels and patterns of mortality amongst the different treatments to be very similar. Randomised block analysis of variance ($\sqrt{}$arcsin transformed data)

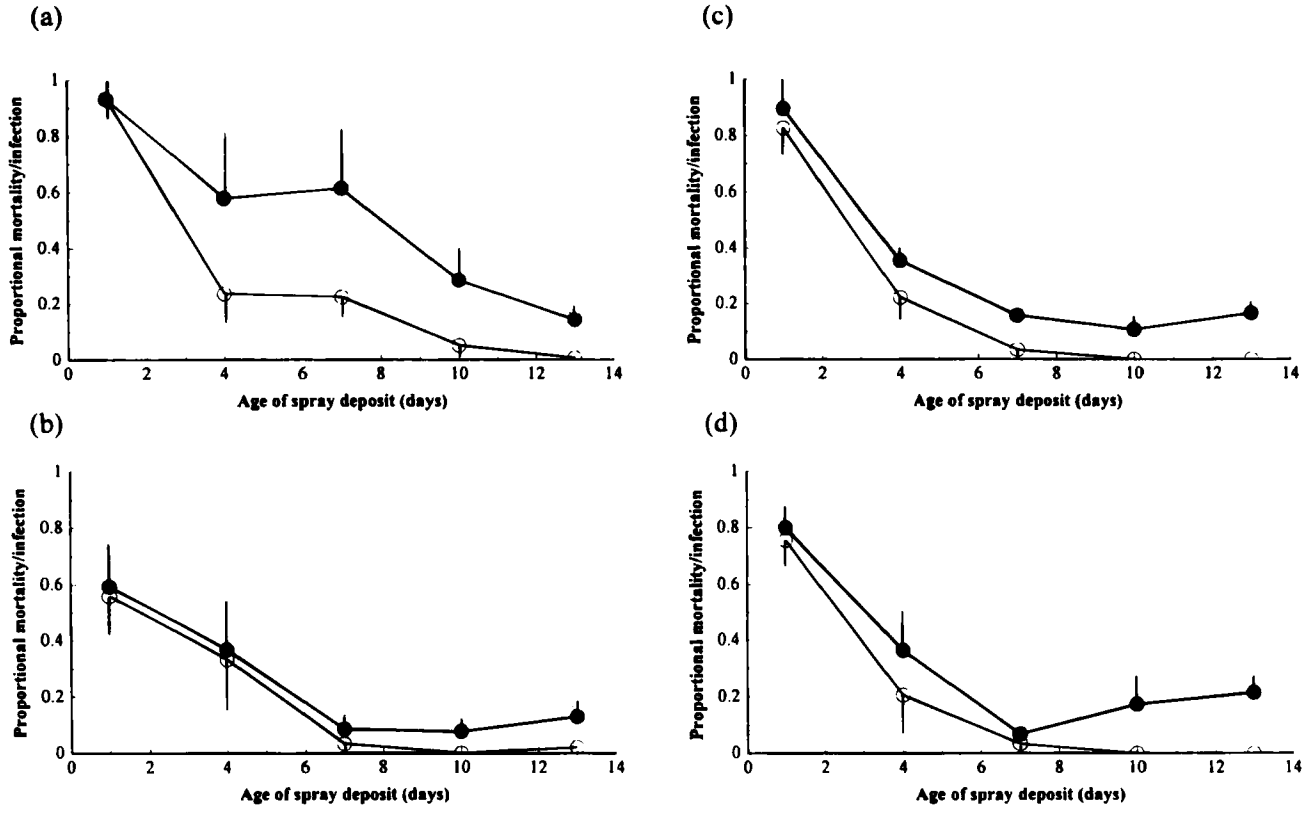

Fig. 2. Mean (±SE) (●) mortality and (○) infection in populations of *Schistocerca gregaria* following secondary pick-up of conidia from different spray residues of (a) ULV, (b) MULV, (c) VLV and (d) knapsack. Experimental techniques are described in the main text.

for each date revealed a significant difference between treatments on the day 7–10 exposure only ($F_{3,9} = 4{\cdot}64$; $P = 0{\cdot}032$).

Mortality was observed to increase towards the end of the experiment in three of the treatments. Examination of the infection data reveal this to be due to increases in background mortality rather than increases in disease levels; infectivity of the spray residues of each treatment showed a steady decline with time. Once again, the only significant difference between treatments occurred on day 7 when infectivity of all but the ULV treatment had fallen to very low levels ($F_{3,9} = 4{\cdot}36$; $P = 0{\cdot}037$). The higher infectivity of ULV was also indicated in the survival analysis for this date; the AST for ULV was significantly lower than for the other treatments ($F_{3,9} = 5{\cdot}579$; $P = 0{\cdot}0193$). There were no significant differences in the survival times between treatments on any other dates.

The consequences of residual infection on total mortality following a single spray application were examined using the host–pathogen model presented in Thomas *et al.*[5] This model describes the total mortality from a spray application by assigning a probability for infection via direct contact with the spray, S, and an instantaneous risk of infection per healthy host per day from the contact with spray residue, r. This risk of infection as a result of the spray residue is described by a negative exponential $r = P\ \exp(-\alpha t)$, where P is a measure of the initial infectivity of the residue and α is a measure of decay rate (see Carruthers *et al.*[9] and Thomas *et al.*[5])

Using this description of r, if we let $H(t)$ = the population of healthy grasshoppers t days after spraying and $H(0) = (1 - S) \times$ (healthy population before spraying), then the equation for $H(t)$ is:

$$H(t) = H(0)\exp[P/\alpha(\exp(-\alpha t) - 1)]. \quad (1)$$

To obtain estimates of the risk of infection, r, exponential regressions were fitted to the mean infection data in Fig. 2 (corrected to give infection risk per day). The exponential decay curves resulting from this are presented in Fig. 3.

In Fig. 4, we use these estimates of r in the host–pathogen model to examine the contribution of residual infection to total mortality 14 days after application (a time when residual infectivity is negligible for all treatments). It can be seen that for each spray type, secondary pick-up of conidia can greatly increase the impact of a spray application. The magnitude of the effect depends on direct spray contact rate; the more efficient the application the smaller the proportion of individuals available for residual infection. However, for low to intermediate contact rates all treatments provide substantial additional mortality. This is most pronounced for the ULV treatment where, due to the high

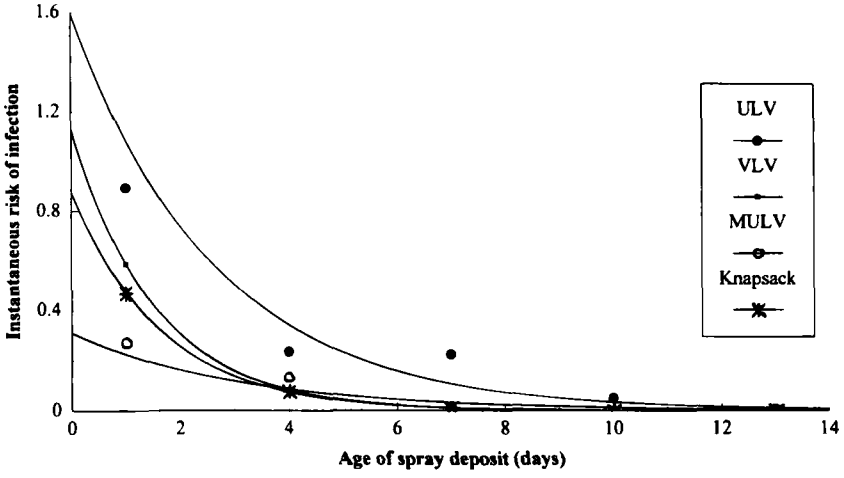

Fig. 3. The instantaneous risk of infection, r, from contact with spray residues of the four spray treatments. These infection profiles were calculated by fitting exponential regressions to the infection data presented in Fig. 2 (corrected to give risk of infection per day). Equations for the different regressions are: $r = 1{\cdot}59 \exp(-0.385\ t)$, $R^2 = 91{\cdot}60$, half life = 1·80 days for ULV; $r = 1{\cdot}12 \exp(-0{\cdot}649\ t)$, $R^2 = 99{\cdot}99$, half life = 1·07 days for VLV; $r = 0{\cdot}31 \exp(-0.320\ t)$, $R^2 = 84{\cdot}12$, half life = 2·17 days for MULV; and $r = 0{\cdot}87 \exp(-0{\cdot}615\ t)$, $R^2 = 99{\cdot}99$, half life = 1·11 days for knapsack.

initial risk of infection from the spray residue, even very low initial spray contact rates result in almost 100% mortality. For the other treatments, spray hit rates in the region of 75–90% are required to obtain mortality levels >95%.

4 CONCLUSIONS AND DISCUSSION

4.1 Virulence of *M. flavoviride* to *S. gregaria* and *Z. variegatus*

The strain of *M. flavoviride* used in this trial has been shown to have high virulence to *S. gregaria* in laboratory bioassays and is the standard strain used in the LUBILOSA project. It has also been the main subject of detailed studies on the production of submerged conidia and was therefore selected for use in this trial. However, due to the relative specificity of this isolate, only low levels of infection were observed in populations of *Z. variegatus*. This species was included in the present study because of its distribution in humid tropical zones where environmental conditions could favour the use of water-based formulations of mycoinsecticides. It is necessary, therefore, that further research into production of submerged conidia of strains more virulent to *Z. variegatus* is conducted in order to determine the full potential of water-based formulations for control of this species. A number of suitable candidate strains of *M. flavoviride* from *Z. variegatus* exist within the LUBILOSA isolate collection. Research in this area is likely to continue given the encouraging results on the efficacy of water formulations obtained with *S. gregaria*.

4.2 Direct spray contact

Examination of the direct spray impact data for *S. gregaria* revealed that the application of aqueous conidia

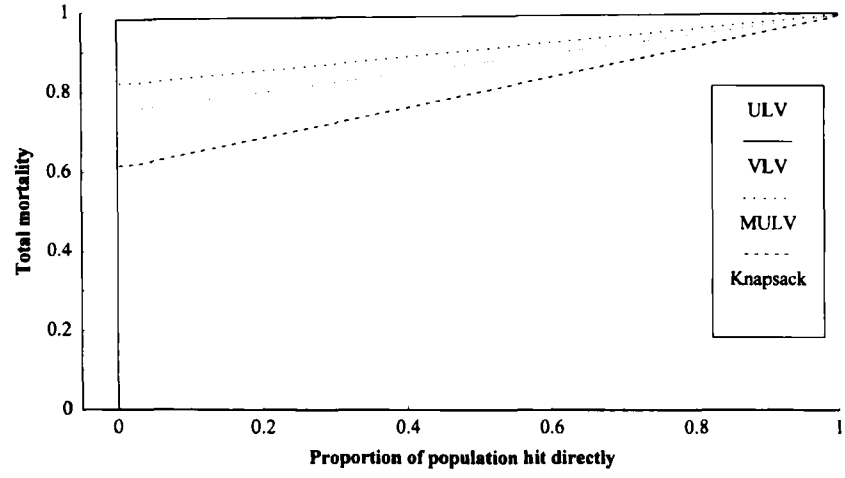

Fig. 4. The effect of secondary pick-up of conidia on total mortality after 14 days with respect to direct initial spray contact. The different lines represent the different residual activities of the four spray treatments.

using a knapsack sprayer gave the shortest AST. Given that the same number of conidia were applied in each treatment, all conidia were equally virulent and all insects were incubated under identical conditions, this result suggests that the high volume application of the knapsack gave better spray penetration of the vegetation resulting in a greater number of conidia actually hitting the target. This effect could be of value in *Z. variegatus* control since many farmers in the humid zone of West Africa have access only to knapsack sprayers and are unable to invest in new ULV application equipment. Additionally, the potentially lower production costs of submerged conidia could make aqueous formulations and high-volume application a viable alternative to oil-based ULV applications of mycoinsecticides in these areas.

4.3 Spray residue

The results of the spray residue experiment reveal that both aerial and submerged conidia can survive long enough in the environment to effect significant mortality after spraying. The half-lives of the different treatments were rather low compared with some previous data (one or two days compared with around six days in Thomas *et al.*[5]). This is likely to be due in part to the lower doses used in the present study ($3{\cdot}5 \times 10^{12}$ conidia ha^{-1} as opposed to 5×10^{12} in Thomas *et al.*)[5]. Differences between studies in a range of biotic (e.g. vegetation structure and host species) and abiotic (e.g. temperature and UV radiation) factors are also likely to cause variability.

In the present study the most persistent treatments were the MULV application of submerged conidia (an aqueous emulsion with 100 ml $litre^{-1}$ oil) and the ULV application of aerial conidia (an oil formulation). These had half-lives about twice as long as the treatments in which oil content was either very low (10 ml $litre^{-1}$) or absent. Enhanced infectivity of conidia in oil formulations has been recorded previously.[11,12] These results on persistence suggest that oil in the formulation may also provide enhanced protection to conidia after spraying. They also indicate that when this protection is provided, there is little difference between survival of conidia produced in submerged culture and those produced on dry substrate in air. However, even though survival was similar, the overall infectivity of the MULV residue was much lower than that of the ULV. This was due to a lower risk of infection immediately after spraying in the MULV treatment (i.e. $P = 0{\cdot}31$ for MULV compared with $P = 1.59$, $P = 1{\cdot}12$ and $P = 0{\cdot}87$ for ULV, VLV and knapsack respectively). The reason for this is not clear but it may relate to the droplet sizes in the initial application. The spray droplet data reveal that the MULV treatment had both fewer and smaller droplets than the other water-based sprays. Although this is expected from the lower application volume and higher speed of the sprayer's spinning disc in this treatment, given that small droplets tend to drift further and evaporate faster, it is possible that this treatment actually received proportionately fewer conidia initially and that penetration of the vegetation by spray droplets was limited. For the ULV treatment, which had smaller droplets still, the protection and enhanced infectivity provided by the oil-based formulation may have compensated for this.

Overall, the residual infectivity of the ULV treatment accounted for much higher mortality than the other treatments; the model predicted that >95% mortality was possible with only minimal hit rates. For the water-based treatments, direct contact rates in the region of 75–90% would be required to achieve such mortality levels. In the small field plots used in the present study, spray efficiency and direct contact rate with conidia was very high, so this limitation was of little consequence. However, as mentioned in the introduction, direct spray contact under different field conditions can be much lower and under these circumstances this limited residual infection would greatly reduce the impact of a water-based spray application. Thus, in systems where direct spray contact is limited due to dense vegetation, or for control of concealed pests such as coffee berry borer and stem borers (i.e. pests where the primary route of infection is likely to be due to residual pick-up rather than direct contact with spray), the increased persistence of conidia in oil formulations may increase the field efficacy of applied microbials.

Under conditions where prolonged activity of the spray residue is considered undesirable however (potentially in some conservation areas, for example), water-based formulations may have an advantage over conventional oil-based applications. Here, efforts to maximise efficiency of the direct spray contact (which could include repeated applications) would be of greatest benefit. However, the use of submerged conidia is not necessarily restricted to application in water-based formulations. The promising results obtained with the MULV treatment suggests that emulsions with a higher proportion of oil are likely to perform similarly to conventional oil-based formulations.

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